IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Dorit PLAT et al.	Confirmation No. 3410
Serial No.:	10/572,782	Examiner: Abigail L. FISHER
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For:	STABILIZED FORMULATIONS OF PHOSPHATIDYLSERINE	

DECLARATION OF Gai Ben-Dror
UNDER 37 C.F.R. § 1.132

In the Matter of
US Patent Application Ser. No. 10/572,782
Stabilized Formulations of Phosphatidylserine (hereafter "**the patent application**")

I, Gai Ben-Dror, hereby declare and state as follows:

I am a Chemical and Biotechnology engineer since the year 1996. I have been employed by Enzymotec Ltd. since the year 2002, and am currently employed as the Director of Process Development of Enzymotec Ltd. My CV is attached hereto (**Annex A**).

I am a co-inventor of the invention subject of the above-identified application.

I have read and understand the patent application. I have also read and understand the amended claims, attached hereto, to be filed with this declaration. I have also read and understood EP 922707, hereafter De Ferra).

The invention recited in amended claims relates to a phosphatidylserine (PS) composition of matter comprising an oil base and from about 1 to about 99% and preferably 45% (w/w) PS, wherein the PS is in the form of its salt with a divalent ion,

which salt is insoluble, and is dispersed (and not dissolved) in the oil base. The PS composition exhibits a stability of less than about 5% decomposition of the PS after a storage period of at least 6 months. The divalent ion is particularly calcium or magnesium.

The PS comprised in the composition of matter of the invention is prepared in an aqueous (monophasic) system, as shown, e.g., in Example 1 of the Declaration by Ms. Neta Scheinman, submitted together with the previous Amendment (at page 2).

The following experiments were conducted at my request and under my direct supervision and control, and demonstrate that unlike PS that is prepared in an aqueous monophasic system as is the PS of the invention, PS prepared in a diphasic reaction system is soluble, and when inserted into an oil base, it forms a solution and not a dispersion,. I participated in the design of and approved all experiments. I continuously monitored the experiments in order to assure that they would be carried out according to their design.

I. Repeating Example 2 of De Ferra et al. (EP 0 922 707), and demonstrating that the resulting PS is soluble in oil.

Preparation of organic phase:

1. 200 g of Epikuron 135F (Cargill, Germany),1500 ml toluene and 50 ml water were placed into a 3000 ml reactor, under nitrogen and the mixture was concentrated under vacuum at 45^0C, distilling about 1000ml of the solvents.

Preparation of aqueous phase:

Another 2000 ml Erlenmeyer was loaded with 11 g calcium chloride, 14 g sodium acetate trihydrate, 700 ml distilled water, 8.5 g acetic acid, 300 g L-serine (Kyowa, Japan) and 0.6 g PLD (Phospholipase D, Meito Sangyo, Japan), that were mixed until dissolved (pH= 4.2).

Diphasic reaction:

1. The two solutions were combined and the resulting mixture was heated to and kept at 25^0C with strong stirring for 24 hr.

2. HPTLC analysis showed that there was no significant progress in the reaction (only 15% PS content of the total phospholipids). Therefore, another 4g of PLD were added and the solution was heated and kept at 40^0C.
3. HPTLC analysis showed a PS content of 48% of the total phospholipids. Therefore another 5 g of PLD were added and the solution was mixed for 3 days.
4. HPTLC analysis showed a PS content of 85% of the total phospholipids.
5. The mixture was then added to a suspension of 13 g decalite in 250 ml toluene and filtered, washing the filter with 200 ml of toluene/water (3/1, V/V).
6. The aqueous phase was separated and the organic phase, after further filtration on decalite, was concentrated under vacuum to an about 200 g residue.
7. 120 g of the residue were taken up into 1300 acetone and stirred for 6 hr at room temperature.
8. After cooling the mixture to 4^0C, the product was filtered to give about 55 g of PS.

Solubility test:

The solubility of the resulting PS was demonstrated in two different ways:

1. 1.2 g PS of the resulting PS were added to 10 ml hexane and the mixture was shaked for 1 min. A clear solution was obtained indicating that the PS is completely soluble in hexane.

2. 23.19 g of the resulting PS and 24 g MCT were dissolved in hexane and concentrated under vacuum to give 47g clear PS fluid, indication that the PS is soluble in oil.

Further testing was undertaken, which demonstrates that producing PS in a diphasic reaction results in a soluble PS:

II. Preparation of fluid PS from soy lecithin (Centrolex FSB):

Preparation of organic phase:

1. 1020 g vegetable lecithin (Centrolex FSB, Solae, USA/Germany) and 6800 ml ethanol 96% were incubated in Erlenmeyer flasks at 20-25^0 C for 2 hr with shaking at 200-250 rpm.

2. The Erlenmeyer content was filtered through Whatman No.41 paper and the extraction (35 g dry basis) was concentrated under vacuum evaporation until a honey-like texture appeared.
3. 500 ml hexane and 260 g MCT oil were added to the extraction and the solvent mixture was evaporated under vacuum until no solvent residue was seen.
4. 500 ml hexane were added twice and evaporated under vacuum, and the resulting lecithin/MCT oil (135g) were dissolved in 300 ml hexane:ethyl acetate (70:30) solution.

Preparation of aqueous phase:
1. 11.25 g calcium chloride and 2.11 g acetic acid were added to 450 ml distilled water, titrated with caustic soda to pH 4.1-4.3, and heated to 40^0C.
2. 150 g L-serine (Kyowa, Japan) and 3 g PLD (Meito Sangyo, Japan) were added and mixed until fully dissolved.

Diphasic reaction:
1. The organic phase was added to the aqueous phase.
2. The resulting diphasic system was stirred for about 48 h at 40^0C under nitrogen environment.
3. The organic phase was separated from the aqueous phase and concentrated under high-vacuum evaporation.
4. The concentrate was added to 500 ml hexane for dry filtration through Whatman GF/A paper and 0.2μm solvent-resistant membrane.
5. The filtrate was concentrated under high-vacuum evaporation to give 132 g **clear** fluid oil containing 20-25% soluble PS calcium salt.

III. Preparation of PS from marine lecithin (PhosphoNorse 60 E322) using hexane as the organic solvent:

Preparation of organic phase:
1. 60 g PhosphoNorse 60 E322 (Eximo AS, Norway) and 60 g of MCT oil were added to 500 ml hexane.
2. The hexane was evaporated under vacuum.

3. 500 ml hexane were added and evaporated again under vacuum and the resulting lecithin/MCT oil (120 g) was dissolved in 300 ml hexane:ethyl acetate (70:30) solution.

Preparation of aqueous phase:

1. 11.25 g Calcium chloride and 2.11 g acetic acid were added to 450 ml distilled water, titrated with caustic soda to pH 4.1-4.3 and heated to 40^0C.
2. 150 g L-serine (Kyowa, Japan) and 5 g PLD (Meito Sangyo, Japan) were added and mixed until fully dissolved.

Diphasic reaction and deoiling:

1. The organic phase was added to the aqueous phase.
2. The resulting diphasic system was stirred for about 20 h at 40^0C under nitrogen environment.
3. The organic phase was separated from the aqueous phase and mixed with 60 g of MCT oil.
4. The mixture was concentrated under high-vacuum evaporation
5. The concentrate was added to 500 ml hexane for dry filtration through Whatman GF/A paper.
6. The filtrate was concentrated under high-vacuum evaporation to give about 115 g residue.
7. The residue was dropwise added into 1L ethanol ABS and stirred for about 20 min at RT.
8. Stirring was stopped and mixture was separated.
9. The upper phase was removed and fresh ethanol ABS was added up to a volume of 1 L.
10. Step 9 was repeated 3 more times and then the mixture was filtered through Whatman No.41 paper.
11. The filtration cake was dried at 30^0C under vacuum for about 12 h to give 30-35 g powder containing 45-50% PS calcium salt.

Solubility test:

1. The powder was dissolved in hexane:ethanol solution (about 80-90% hexane and 10-20% ethanol ABS) and added to MCT oil in a 1:1 ratio.
2. The solution was concentrated under high-vacuum evaporation to 20-25% PS calcium salt completely dissolved in 60-70 g fluid.

IV. Preparation of PS from marine lecithin (PhosphoNorse 60 E322) using toluene as the organic solvent:

Preparation of organic phase:

1. 35 g PhosphoNorse 60 E322 (Eximo AS, Norway) were added to 600 ml toluene.
2. The toluene was evaporated under vacuum until 50-70 ml of residue.
3. Fresh toluene was added up to 200ml total volume.

Preparation of aqueous phase:

1. 13.13 g Calcium chloride and 2.46 g acetic acid were added to 525 ml distilled water, and titrated with caustic soda to pH 4.1-4.3.
2. The aqueous solution was heated to 40^0C and 175 g L-serine (Kyowa, Japan) and 2.9 g PLD (Meito Sangyo, Japan) were added and mixed until fully dissolved.

Diphasic reaction and deoiling

1. The organic phase was added to the aqueous phase
2. The resulting diphasic system was stirred for about 20 h at 40^0C under nitrogen environment.
3. The organic phase was separated from the aqueous phase, and concentrated under vacuum evaporation to 90 ml residue.
4. The residue was taken up into fresh 100 ml toluene for dry filtration through 1µm solvent resistant membrane.
5. The filtrate was concentrated under vacuum evaporation to an about 90 ml residue
6. The residue was dropwise added into 1.16 L of ethanol ABS and stirred for about 20 min at RT.
7. Stirring was stopped and mixture was separated.

8. The upper phase was removed and fresh 600 ml ethanol ABS was added and stirred for about 20 min at RT.
9. Stirring stopped and the mixture was separated.
10. The upper phase was removed and fresh 600 ml toluene were added to completely dissolve the powder.

Solubility test:
18.1 g MCT oil were added to give a fluid form, concentrated under high-vacuum evaporation to give 50 g of clear fluid containing 20-25% PS calcium salt.

The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing from the application referenced herein.

Date: _____ By: _____

Gai Ben-Dror